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Combined Effect of Two Different Polymorphic Sequences Within the β Globin Gene Cluster on the Level of HbF

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β thalassemia and Hb Lepore heterozygotes included in this study exhibit fetal hemoglobin levels varying from trace quantities to 14% (1.74 g/dl) of total hemoglobin in the adult. In this work, we have examined the correlation of DNA sequence polymorphisms with the observed HbF level. The analysis of polymorphic markers within the β globin cluster in 39 individuals heterozygous for β thalassemia or Hb Lepore confirms the previous findings for homozygous β thalassemia: the presence of both an $(AT)_9$ T_5 sequence configuration at position −540 of the β globin gene and a (C → T) variation at −158 of the Gγ globin gene is associated with elevated expression of HbF. However, at least one defective β globin gene is required to reveal this association. The best evidence is from the study of individuals heterozygous for Hb Lepore with various levels of HbF. In these individuals it was possible to explore the effect of a single $(AT)_x$ T_y motif (the other being absent from the rearranged Lepore chromosome) on HbF expression. The presence of the $(AT)_9$ T_5 configuration increases HbF level from a median of 0.515 g/dl observed in $(AT)_7$ T_7 subjects, to 1.39 g/dl.

We confirm the existence of linkage disequilibrium between the (C → T) variation at −158 of Gγ gene and the $(TG)_{13}$ configuration at the second intervening sequence (IVS-2) of Aγ gene and identify two new polymorphisms in this region: $(TG)_7$ $(CG)_5$ $(TG)_8$ linked to haplotype V and $(TG)_8$ $(CG)_5$ $(TG)_{10}$ linked to haplotype II. This study suggests that two distinct regions of the β cluster, whether *in cis* or *in trans* to each other, can interact to enhance HbF expression when a β thalassemic determinant is present in heterozigosity. Am. J. Hematol. 57:269–276, 1998.

Key words: polymorphisms; β globin gene cluster; expression; HbF

INTRODUCTION

In normal adults, synthesis of fetal hemoglobin (HbF) persists at very low levels (<0.6%) and is confined to a subpopulation of erythrocytes defined as F cells. There are various genetically different conditions in which higher levels of HbF synthesis can persist in adult life. These are referred to as hereditary persistence of fetal hemoglobin (HPFH). Rare forms of this condition, with

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high increase in HbF levels in heterozygotes (5–25%), exhibit a clear Mendelian inheritance and are caused by extensive deletions within the β globin cluster or by point mutations in the γ globin gene promoters. On the other hand, a common form of nondeletion HPFH, named Swiss type HPFH, is characterised by only a small increase in HbF (1–3% in normal adults) with a heterocellular distribution and by a complex inheritance pattern. The genetic determinant of this condition behaves as an allele of the β globin gene cluster in some families [1–3] whereas in others it segregates independently of this cluster [4–11].

Among the polymorphic genetic markers of the β globin gene cluster, a common C → T variation at −158 of the Gγ globin gene (detected as a *Xmn*I restriction fragment length polymorphism) has been associated with a modest increase in the HbF level in normal individuals [2,5,6], and with an enhancement of the HbF under the erythropoietic stress of homozygous β-thalassemia and sickle cell anemia [12–14]. High HbF levels in homozygous β thalassemia have also been associated with sequence polymorphisms, specifically at the −540 region of the β globin gene [15]. In that study, both the C → T variation at −158 of Gγ gene and the $(AT)_9$ T_5 configuration near −540 of the β globin gene have been associated with an unusually high HbF level. This $(AT)_9$ T_5 configuration had previously been associated with a silent β thalassemia phenotype [16]. However, other studies failed to show any functional significance of this sequence configuration in nonanemic individuals [17].

These disparate data, obtained for two extremes of the phenotype (nonanemic state and homozygous β thalassemia), prompted us to explore the involvement of these putative genetic determinants linked to the β globin gene cluster in the expression of HbF, in mildly anemic heterozygous β thalassemia individuals. We also studied a group of Hb Lepore (Baltimore) carriers with various levels of HbF. As they have only one copy of the $(AT)_x$ T_y motif, the other being deleted in the rearranged Lepore chromosome, it was possible to explore the effect of a single $(AT)_x$ T_y motif on HbF expression.

We confirm the previously observed genetic association between these two β globin gene cluster markers and the expression status of the HbF [12–14]. The effect on HbF was observed even when particular alleles at the two marker sites were *in trans* to each other. This *in trans* effect probably contributes to the complex inheritance pattern usually observed in the heterocellular HPFH condition.

MATERIALS AND METHODS

Subjects

This study included 39 individuals (older than 5 years of age) belonging to 16 families. There were 26 β thalassemia heterozygotes, 7 Lepore (Baltimore) carriers, and 6 nonanemic individuals with normal hematological indices. The range of HbF level varied from trace quantities to 14% (1.74 g/dl).

Hematological and Hemoglobin Analysis

Hematological indices were determined using an automated cell counter. HbF was quantified by a HPLC procedure [18] and DEAE cellulose chromatography was used to measure the HbA_2 level [19]. The gamma globin chain ratio was determined by reverse-phase HPLC [20].

DNA Preparation and Haplotype Analysis

DNA was prepared from peripheral blood leukocytes by a salting-out extraction procedure [21] and further purified by two cycles of phenol-chloroform extraction. The haplotype of the β globin gene cluster was determined either by Southern blotting (for *Hin*d III site at the IVS-2 of Gγ and Aγ genes) or by the previously described PCR-based assay [22]. The latter was employed for studying the polymorphism of the following sites: *Hin*c II 5′ of the ε gene, *Xmn* I 5′ of the Gγ gene, two *Hin*c II sites in the Ψβ region, *Rsa* I site 5′ of the β gene, *Ava* II in the IVS-2 of the β gene, and *Hin*f I site 3′ of the β gene. The haplotypes were named according to Antonarakis et al. [23].

Direct DNA Sequencing

For determining the sequence configuration of the $(AT)_x$ T_y motif, a 740-bp fragment from the 5′ region of the β globin gene was amplified and sequenced as described elsewhere [24]. For individuals heterozygous for the polymorphism of *Rsa* I site, allele specific DNA sequencing was performed as described earlier [15]. The analysis of the polymorphic motif $(TG)_x$ $(CG)_n$ $(TG)_y$ at IVS-2 of the Aγ gene was performed by DNA amplification and sequencing with the primers described by Lanclos et al. [25].

Characterization of Thalassemia and Lepore Mutations

Screening for β thalassemia mutations was performed by ARMS (Amplification Refractory Mutation System) [26], using primers specific for the most frequent mutations in the Portuguese population [27], or by direct sequencing of the amplified DNA. The Lepore alleles were characterised by PCR followed by digestion with *Pvu* II and *Ava* II, as described by Camaschella et al. [28]. Absence of deletion(s) in the α globin gene cluster in all the studied individuals was ascertained by Southern blot analysis of *Bam* HI and *Bgl* II digestions [29].

Statistical Analysis

Due to the small size of samples in each genotypic group, the non-parametric Wilcoxon-Mann-Whitney test

TABLE I. Summary of Hematological and Genetic Data on the β^{thal} Trait, Hb Lepore Trait, and Normal Individuals Included in the Study*

		Subject (haplotype)[a]	Sex (age)	Mutation	Hb (g/dl)	HbF %	HbF g/dl	HbA_2 (%)	Gγ (%)	$A\gamma^T$ (%)	*Xmn* I (−158Gγ)	$(AT)_x$ T_y x/y	$(AT)_x$ T_y x/y
Fam.A	I-1	(VI/H Indian)[b]	M (40)	CD15	13.8	1.2	0.17	5.2	50	50	−/−	7/7	9/5
	I-2	(IX/IV)	F (34)	—	14.0	1.2	0.17	2.2	69	0	+/+	7/7	7/7
	II-1	(IX/H Indian)[b]	M (10)	CD15	11.2	7.3	0.82	3.8	67	20	+/−	7/7	9/5
Fam.B	I-1	(IX/I)[b]	M (42)	CD39	11.3	4.2	0.47	3.5	66	0	+/−	7/7	7/7
	I-2	(I/III)	F (41)	—	13.3	0.3	0.04	2.4	53	0	−/+	7/7	9/5
	II-1	(III/I)[b]	M (15)	CD39	11.9	7.0	0.83	4.2	62	0	+/−	9/5	7/7
Fam.C	I-1	(I/VI)[b]	M (46)	IVS-I-6	13.2	1.0	0.13	5.1	nd	nd	−/−	7/7	7/7
	I-2	(I/II)[b]	F (38)	CD39	10.0	1.6	0.16	7.0	38	31	−/−	7/7	7/7
	II-1	(I/VI)[b]	M (11)	IVS-I-6	9.7	Traces	Traces	5.9	nd	nd	−/−	7/7	7/7
Fam.D	I-1	(IV/V)[b]	M (Adult)	IVS-I-1	13.4	Traces	Traces	5.6	nd	nd	+/−	7/7	7/7
	I-2	(I/V)[b]	F (Adult)	IVS-I-1	11.3	Traces	Traces	5.5	nd	nd	−/−	7/7	7/7
	II-1	(I/V)[b]	M (>5)	IVS-I-1	10.6	1.0	0.11	5.1	nd	nd	−/−	7/7	7/7
Fam.E	I-1	(III/II)[b]	M (46)	CD39	11.7	6.0	0.70	5.1	63	23	+/−	9/5	7/7
	I-2	(II/V)	F (44)	—	13.4	0.4	0.05	3.0	42	42	−/−	7/7	9/5
	II-1	(II/II)[b]	M (7)	CD39	10.1	Traces	Traces	5.1	47	53	−/−	7/7	7/7
	II-2	(II/II)[b]	F (7)	CD39	10.1	Traces	Traces	5.4	40	60	−/−	7/7	7/7
	II-3	(V/II)[b]	F (15)	CD39	11.0	1.0	0.11	5.0	32	38	−/−	9/5	7/7
Fam.F	I-1	(3 Chinese/I)[b]	F (41)	CD39	10.6	4.5	0.48	4.1	nd	nd	−/−	7/7	9/5
	I-2	(I/III)	M (44)	—	16.2	0.8	0.13	nd	nd	nd	−/+	9/5	7/7
	II-1	(III/I)[b]	F (21)	CD39	10.9	5.6	0.61	4.1	60	0	+/−	7/7	9/5
Fam.G	I-1	(IX/V)[b]	M (66)	IVS-I-1	10.3	3.3	0.34	5.4	nd	nd	+/−	7/7	7/7
	II-1	(III/V)[b]	M (31)	IVS-I-1	12.8	3.8	0.49	6.8	62	0	+/−	9/5	7/7
	II-2	(V/II)	F (30)	—	13.2	0.7	0.09	2.3	65	14	−/−	9/5	7/7
	III-1	(V/V)[b]	M (5)	IVS-I-1	9.7	4.7	0.46	6.1	39	0	−/−	9/5	7/7
Fam.H	I-1	(III/II)[b]	M (Adult)	CD39	12.7	0.1	0.01	5.4	nd	nd	+/−	7/7	7/7
	I-2	(nd/VI)[b]	F (Adult)	IVS-I-6	10.7	0.2	0.02	5.4	24.7	0	−/−	7/7	7/7
	II-1	(nd/II)[b]	F (>5)	CD39	9.1	1.6	0.15	6.0	nd	nd	−/−	7/7	7/7
Fam.I	I-1	(V/III)[b]	F (69)	Lepore Baltimore	12.4	14	1.74	2.5	nd	nd	−/+	9/5	del
	I-2	(I/V)	M (69)	—	14.9	0.6	0.09	3.5	nd	nd	−/−	7/7	9/5
	II-1	(V/III)[b]	F (23)	Lepore Baltimore	12.0	14	1.68	2.0	63	0	−/+	9/5	del
Fam.J	I-1	(V/III)[b]	M (53)	Lepore Baltimore	11.9	9.0	1.10	2.3	nd	nd	−/+	9/5	del
	II-1	(V/III)[b]	M (22)	Lepore Baltimore	12.6	5.0	0.63	2.6	nd	nd	−/+	9/5	del
Fam.K	I-1	(I/III)[b]	F (57)	Lepore Baltimore	12.5	4.3	0.54	1.4	nd	nd	−/+	7/7	del
	II-2	(I/III)[b]	F (52)	Lepore Baltimore	11.9	4.1	0.49	1.6	nd	nd	−/+	7/7	del
Individual cases	1	(IX/III)[b]	M (63)	Lepore Baltimore	15.0	4.0	0.6	1.7	67	0	+/+	7/7	del
	2	(nd/II)[b]	M (Adult)	CD39	12.9	Traces	Traces	4.5	66.5	19.5	+/−	7/7	7/7
	3	(III/III)[b]	F (Adult)	IVS-I-1	10.4	6	0.62	4.5	77.5	0	+/+	9/5	7/7
	4	(3 Chinese/I)[b]	F (Adult)	IVS-I-110	11.7	Traces	Traces	5.0	nd	nd	−/−	7/7	7/7
	5	(4 Black/I)[b]	F (Adult)	CD39	10.6	0.8	0.08	5.6	nd	nd	−/−	7/7	7/7

*nd = not determined; del = deleted; M = male; F = Female.
[a]Haplotypes according to Antonarakis et al. [23].
[b]β^{thal} or Lepore chromosome.

[30] was used. The parametric *t*-test [31] was also used to evaluate which test was accurate. Levene's test for homogeneity of variance was used to evaluate the accuracy of the *t*-test.

RESULTS

The hematological and genetic data obtained in this study are shown in Table I. The Gγ and Aγ values excluded the presence of major gene deletions and nondeletional forms of HPFH usually associated with the production of relatively high levels of HbF in heterozygotes. The phase of the different restriction site polymorphisms and of the $(AT)_x$ T_y motif was ascertained by family studies (only informative families were considered).

Among a total of 51 independent chromosomes (from all the unrelated parents studied), 10 different haplotypes were observed. One of them was exclusively linked to a β thalassemic gene (H Indian), five either to the β^A or the β thalassemic genes (I, II, III, V, VI), and the remaining four to the β^A gene (IV, IX, 3 Chinese, 4 Black) (Table I). No deletional α thalassemia was found (data not shown). Similarly, no correlation was observed between the classical haplotype (according to Antonarakis et al. [23]) or the nature of the β thalassemia mutation and the level of HbF (Table I).

TABLE II. Distribution of the Extended 5′ Subhaplotypes and $(AT)_x$ T_y Motifs Within the β Globin Gene Cluster in Chromosomes Carrying a β Thalassemia or Lepore ($\beta^{T/L}$) or a Normal (β^A) Globin Gene

A. Distribution of the extended 5′ subhaplotypes

Class	Classical 5′-subhaplotypes[a]	*Xmn* I (−158Gγ)	IVS2 (Aγ)	Chromosomes β^A (%)	Chromosomes $\beta^{T/L}$ (%)
A	− + − + + (III,IV,IX)	+	$(TG)_{13}$	12 (37)	5 (26)
B	+ − − − − (I,V)		[b]	14 (44)	7 (37)
	− + + − + (II, 3Chinese, H Indian)	−	[c]	4 (13)	5 (26)
	− + + − − (VI)		[d]	1 (3)	2 (11)
	− − − − + (4 Black)		[d]	1 (3)	0
Total				32	19

B. Allele distribution at the $(AT)_x$ T_y site

$(AT)_x$ T_y (5′β)	Chromosomes β^A (%)	Chromosomes $\beta^{T/L}$ (%)
7/7	22 (67)	13 (87)
9/5	11 (33)	2 (13)
Total	33	15

[a]Polymorphic sites are for the enzymes *Hin*c II (ε), *Hin*d III (Gγ and Aγ), and *Hin*c II (ψβ), according to Antonarakis et al. [23].
[b]$(TG)_9$ $(CG)_5$ $(TG)_8$; $(TG)_7$ $(CG)_5$ $(TG)_8$.
[c]$(TG)_8$ $(CG)_5$ $(TG)_{10}$; $(TG)_{10}$ $(CG)_4$ $(TG)_7$.
[d]$(TG)_{10}$ $(CG)_5$ $(TG)_7$.

We have determined the configuration of the region of simple sequence, which extends from position 591 through 658 of Aγ IVS-2. We have confirmed the existence of $(TG)_{13}$ sequence in linkage disequilibrium with the presence of the *Xmn* I site at −158 of Gγ gene and with the 5′ subhaplotypes III, IV, and IX (Table II). Two new polymorphic sequences, $(TG)_7$ $(CG)_5$ $(TG)_8$ linked to haplotype V, and $(TG)_8$ $(CG)_5$ $(TG)_{10}$ linked to haplotype II, have been identified. Polymorphic sequence $(TG)_{10}$ $(CG)_4$ $(TG)_7$ has been found linked to haplotypes 3 Chinese and H Indian, the sequence $(TG)_{10}$ $(CG)_5$ $(TG)_7$ linked to haplotype 4 Black and to haplotype VI.

We explored the effect of 5′ subhaplotype and $(AT)_x$ T_y on the expression status of HbF in all these β thalassemia and Hb Lepore traits. Only 5 of the 20 possible 5′ subhaplotypes were observed in our series, indicating, as previously noted, a nonrandom association among the 5′ subset of polymorphic markers. We, therefore, divided the chromosomes into two classes, according to their extended 5′ subhaplotypes: class A, in which the *Xmn* I site was present and class B in which it was absent (Table IIA). A superscript T/L indicates the presence of a thalassemic/Lepore allele *in cis* or *in trans* to each chromosomal profile.

At position −540 of the β globin gene, only two sequence configurations of $(AT)_x$ T_y motif were observed: $(AT)_9$ T_5 and $(AT)_7$ T_7 (Table IIB). Interestingly, in all the chromosomes bearing the $(AT)_9$ T_5 sequence the *Rsa* I site was constantly present, but not all the chromosomes having the *Rsa* I site bore the $(AT)_9$ T_5 configuration. The HbF levels found in each genotypic group, are summarized by box plots [30] in Figure 1. In order to compare HbF levels between 5′ subgenotypes, and between $(AT)_x$ T_y genotypes, due to the samples' sizes being small, the non-parametric Wilcoxon-Mann-Whitney test [30] was used. We have also performed the parametric *t*-test to evaluate if results would be substantially different. Results of both methodologies revealed to be consistent. In the group of individuals with normal hematological and hemoglobin values, irrespective of their 5′ subgenotypes (AA, AB and BA) (Fig. 1A) and $(AT)_x$ T_y genotype (Fig. 1B), the HbF level was low (median of 0.09 to 0.17 g/dl).

The homozygous state for the class B profile (5′ subgenotype BB^T) was associated with low HbF levels (median of 0.11 g/dl, see Fig. 1A). The presence of the class A profile was associated with higher levels of HbF (Fig. 1A). Statistical comparison of BB^T vs. AB^T and BB^T vs. BA^L shows significant differences (Mann-Whitney's *P* values of 0.0125 and 0.0006, respectively). This effect was observed whether *in cis* to a Lepore gene (5′ subgenotype BA^L, HbF of 1.39 g/dl), or *in trans* to a β thalassemic gene (5′ subgenotype AB^T, HbF level of 0.49 g/dl, Fig. 1A). Profiles AB^T or BA^L do not show differences at a significance level of 5% (Mann-Whitney's *P* value of 0.0675).

The motif $(AT)_x$ T_y was more tightly associated with the HbF level than the extended 5′ subhaplotype (class A or class B profile). In Figure 1B, we observe that genotype 7/7 7/7 was associated with HbF levels clearly dif-

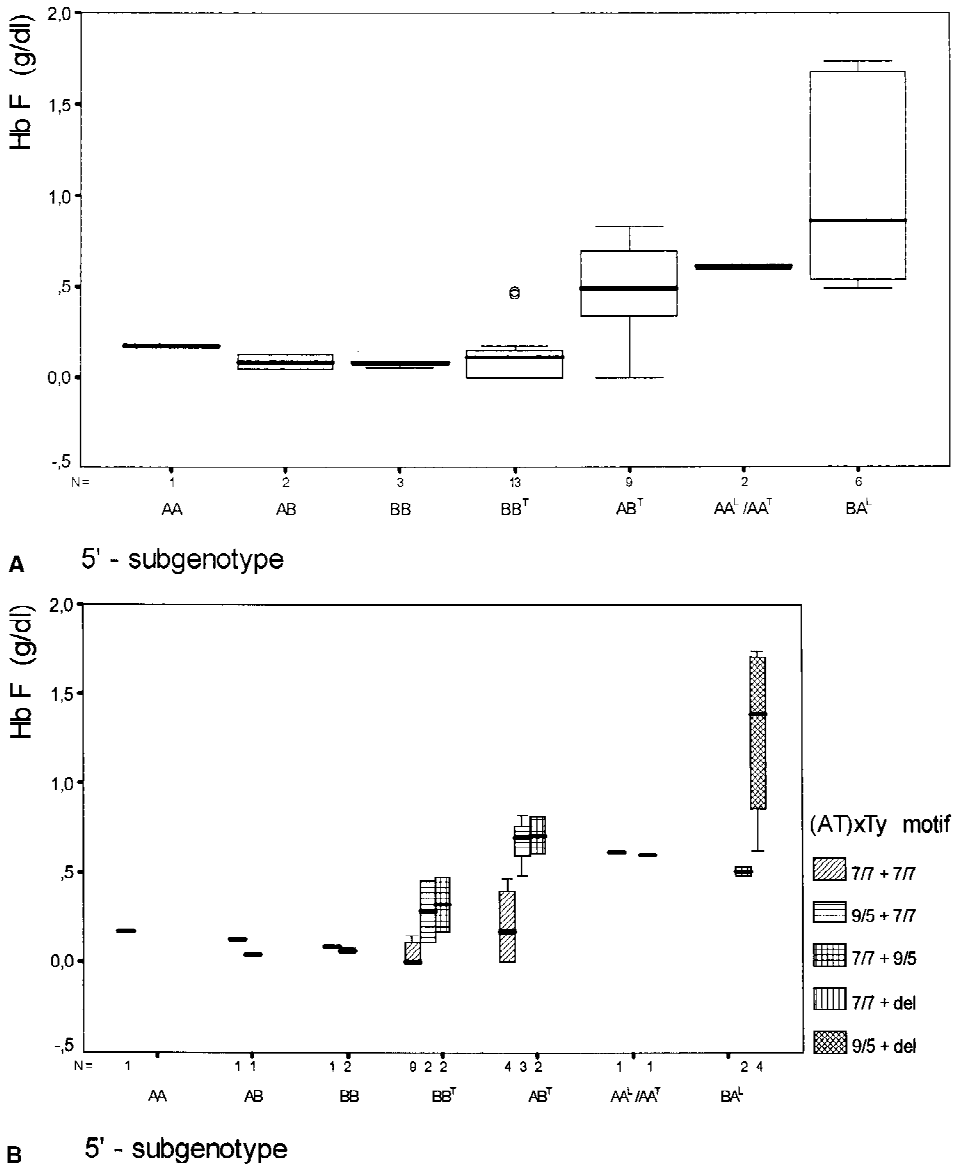
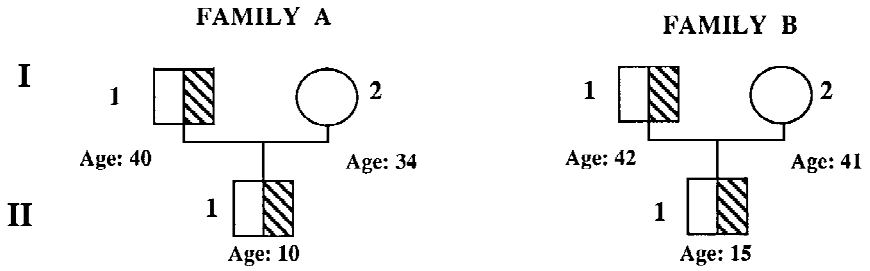


Fig. 1. Absolute fetal hemoglobin (HbF) levels in 23 β thalassemia (BB^T, AA^T) and 7 Hb Lepore (AA^L, BA^L) carriers and 6 normal subjects (AA, AB, BB). A: On the left side are the data for 6 normal individuals categorized in three 5′ subgenotypes (AA, AB, and BB). On the right side are data for 23 β thalassemia carriers and 7 Hb Lepore carriers categorized in four 5′ subgenotypes BB^T, AB^T, BA^L, AA$^{T/L}$. The HbF levels are summarized by box-plots. B: Effect of the (AT)$_9$ T$_5$ allele on the increase of median HbF level in subjects carrying the 5′ subgenotypes BB^T, AB^T, and BA^L. N = number of individuals in each class.

ferent from all other combinations of alleles in this *locus.* The presence of the 9/5 allele and a β thalassemic gene (BB^T, AB^T, Fig. 1B) increases the median HbF level (7/7 7/7 vs. 9/5 7/7, $P = 0.0028$ and 7/7 7/7 vs. 7/7 9/5, $P = 0.0055$) and the effect does not show differences at a significance level of 5%, either *in cis* or *in trans* with the 7/7 allele (9/5 7/7 vs. 7/7 9/5, $P = 0.8312$). All individuals heterozygous for Hb Lepore, identified in this work as the Baltimore type, had the class A profile linked to this mutant allele (AA^L, BA^L). Although the levels of HbF expression in these individuals were heterogeneous (Fig. 1A), an effect of the 9/5 allele is observed (Fig. 1B). Increase in HbF level is from a median of 0.515 to 1.390 g/dl (7/7 del vs. 9/5 del, $P = 0.0339$). The association of these polymorphic markers and the pattern of HbF expression in two families was particularly striking (Fig. 2). In family A, both parents had low HbF levels (0.17 g/dl) whereas the son had 0.82 g/dl of HbF. The son inherited the β thalassemic gene linked to the (AT)$_9$ T$_5$ sequence from his father and the class A profile from his mother.

Family B is also of interest in that the father, a β^{thal} carrier, homozygous for (AT)$_7$ T$_7$ and with an B^{T}A 5′



	Subject	5' Subgenotype	$(AT)_x T_y$	HbF (g/dl)	HbF (%)
Fam.A	I - 1	* B	$(AT)_9 T_5$	0,17	1,2
		B	$(AT)_7 T_7$		
	I - 2	A	$(AT)_7 T_7$	0,17	1,2
		A	$(AT)_7 T_7$		
	II - 1	* B	$(AT)_9 T_5$	0,82	7,3
		A	$(AT)_7 T_7$		
Fam.B	I - 1	** B	$(AT)_7 T_7$	0,47	4,2
		A	$(AT)_7 T_7$		
	I - 2	B	$(AT)_7 T_7$	0,04	0,3
		A	$(AT)_9 T_5$		
	II - 1	** B	$(AT)_7 T_7$	0,83	7
		A	$(AT)_9 T_5$		

Fig. 2. Configurations of the polymorphic markers *Xmn* I 5′ Gγ and $(AT)_x T_y$ 5′ β and HbF levels in two informative families. *β^{thal} chromosome with CD15(TG*G*→TG*A*) mutation. **β^{thal} chromosome with CD39(C→T) mutation.

subgenotype had 0.47 g/dl of HbF. His β^{thal} gene was *in trans* to the class A profile whereas the son, also a β^{thal} carrier, aged 15 years, had 0.83 g/dl of HbF, almost twice the level of his father. He inherited both the $(AT)_9$ T_5 configuration and the class A profile from his mother. Despite the copresence of both $(AT)_9$ T_5 and BA 5′ subgenotype in the mother, her HbF level was very low (0.04 g/dl) as her two β globin genes were normal.

DISCUSSION

In individuals heterozygous for β thalassemia or for Hb Lepore, we have found a strong association between moderately elevated HbF levels, the 5′ configuration in the γ globin gene region with the T allele at −158 Gγ gene, which is a marker of a chromosomal region with extended 5′ subhaplotype A (class A profile) and the $(AT)_9$ T_5 allele at 5′ β globin gene either *in cis* or *in trans.*

This is particularly well illustrated in two family studies (Fig. 2) where the combined effects of the extended 5′ subhaplotype A and the $(AT)_9$ T_5 allele are associated with HbF levels 1.5–5-fold higher than those observed in individuals lacking this genetic arrangement. Both markers were present in chromosomes without β thalassemia and fail to exhibit an association with elevated HbF level (Fig. 1A). However, it must be noted that low levels of HbF in normal individuals are not very precise and narrow differences that may exist could go unnoticed. The determination of the F cell number might have been an alternative to solve this problem but new blood samples were not available for such studies.

The association of a moderate increase in HbF, with certain polymorphic configurations in the Gγ-Aγ-Ψβ region of the β cluster, has not always been straightforward. In the so-called Swiss type HPFH where the HbF level is moderately high, it has been difficult to assess the contribution of β cluster-dependent factors. Although other factors are certainly involved, our data suggest that the classical haplotype is not sufficient to draw conclusions about the contributions of β cluster-dependent factors to the HbF level. By extending the haplotype to the above-mentioned genetic markers, namely the $(AT)_x$ T_y sequences and the C → T variation at −158 Gγ gene, it becomes clear that an association exists between certain sequence configurations and the ''High F'' phenotype. If

a β thalassemic chromosome carries both the $(AT)_9$ T_5 allele and the T allele at −158 Gγ gene, it might appear that the HPFH determinant is linked to the β globin gene cluster. The determination of the $(AT)_x$ T_y configuration is, thus, very important for such an assignment. If, in an individual, these two sequence configurations are *in trans* (Fig. 2, Family A), in the absence of information about the $(AT)_x$ T_y motif, the segregation of the HPFH determinant with the β globin gene cluster will not be apparent, thus leading to the inference of a non-β cluster-dependent factor.

The major contribution of the $(AT)_9$ T_5 configuration to the high HbF phenotype observed in this study, extends previous results from a Sicilian study of homozygous β thalassemia cases [15]. A very mild thalassemia phenotype has been associated with the presence of this configuration, as opposed to $(AT)_7$ T_7. This polypurine-pyrimidine repeat sequence is localized in a region that behaves as a silencer in transient transfection assays with K562 cells [32, 33]. Binding of a putative repressor protein, BP1, is strongly enhanced in the presence of the $(AT)_9$ T_5 configuration. In another pathophysiological model, sickle cell anemia, a correlation between the presence of $(AT)_9$ T_5 and level of β^S globin production in AS individuals from different ethnic groups has also been established [34].

The contribution of the region encompassing Gγ-Aγ-φβ genes to the high HbF phenotype is also of interest. At AγIVS-2 we have observed a non-random association of the specific configuration $(TG)_{13}$ to the presence of *Xmn* I site and the corresponding high HbF level. This subchromosomal profile has been found in individuals with Senegal and Arab India β^S haplotypes, constantly associated with elevated levels of HbF [24]. Sicilian β thalassemics with higher HbF exhibit the same extended 5′ subhaplotype [14].

Our results suggest that certain chromosomal backgrounds create an environment favoring the stimulation of fetal gene expression under the moderate anemic stress associated to β thalassemia or Lepore carriership. It is possible that common factor(s) might mediate this effect. The nuclear protein BP1, which binds to the $(AT)_x$ T_y region at −540 β globin gene, has also been shown to bind to a sequence in the intron 2 of the γ genes [35]. However, in such marker association studies we cannot totally exclude the possibility that other critical regulatory sequences independently linked to the $(AT)_x$ T_y motif and *Xmn* I polymorphism could play a causal role in the regulation of HbF expression.

Our study highlights the importance of the genetic contribution of the β globin gene cluster to HbF expression in both homozygous and heterozygous β thalassemia. It further stresses the complexity that may arise in genetic analysis when the determinants for a given phenotype are nonallelic and the effects are additive. However, given the limited number of subjects investigated in this report, further studies are required to confirm these observations.

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